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High Calcium Zones at the Poles of Developing Medaka Eggs

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Abstract. We have injected medaka fish zygotes with recombinant aequorin and visualized the resulting patterns of luminescence to reveal patterns of free calcium during early development. We have co-injected fluorescein-labeled aequorin to correct for nonuniformities in aequorin (as opposed to calcium) distributions by visualizing the resulting patterns of fluorescence as opposed to luminescence. We have also coinjected a calcium buffer to facilitate calcium diffusion, dissipate apparent calcium gradients, and thus confirm their reality.

An exploratory study shows zones of elevated free calcium at the vegetal as well as the animal pole during the first day of development and thus up to the beginning of gastrulation. A closer study during the first 6 h, and thus through ooplasmic segregation and early cleavage, shows a steady zone of high calcium at the vegetal pole and a slowly oscillating one at the animal pole. The latter is particularly strong during ooplasmic segregation and cytokinesis. This report contains the first unambiguous evidence of relatively steady zones of high cytosolic calcium during the development of an animal egg.

Introduction

Pattern formation starts at the poles—particularly at the vegetal pole—of animal eggs. One striking example of this general statement is in the ascidian egg. Here the unfertilized egg is so free of pattern that any half will form a tadpole; yet after fertilization and the resultant contraction of the cortex toward a region near the vegetal pole, only vegetal fragments will form tadpoles (Reverberi, 1971; Jeffery, 1984; Speksnijder *et al.*, 1990a). Another example is the frog, in which pattern starts as the mitochondrial cloud, the germ plasm, and one or more potent m-RNAs all attach to the vegetal pole of the very young oocyte; in which the zygote's animal half is still totipotent; and in which early blastula cells derived from the vegetal pole plasm induce formation of both the embryonic mesoderm and the dorsal axis (Capco and Jeffery, 1982; Nieuwkoop, 1985; Wylie *et al.*, 1985; Forristall *et al.*, 1991; Melton, 1991). Yet another example is the *Drosophila* egg, in which early pattern is organized from both poles and in which certain key m-RNAs, as well as the germ plasm, move within the maturing oocyte to the insect equivalent of the vegetal pole (Nüsslein-Volhard and Roth, 1989; Ephrussi *et al.*, 1991).

In all three of these eggs—ascidian, frog, and fly—early patterning involves a striking movement of key materials to, or near, the vegetal pole. In these animal eggs, more is known about these key materials than about the mechanisms that establish this pole and drive them to it; the reverse is true in the fucoid egg. Little is known of the key materials in this plant egg; however there is strong evidence that the establishment of a steady subsurface zone of elevated calcium is essential to the mechanism that establishes the first—in this case rhizoidal—developmental pole. Moreover, such a zone has been directly visualized at a stage when localization of the rhizoidal pole is still reversible (Jaffe, 1990a, b).

The role of high calcium zones in the polarizing fucoid egg suggests that they may play a comparable one in animal eggs. Indeed, there is interesting evidence for just such a role in *Xenopus* oocytes (Robinson, 1979; Larabell and Capco, 1988) as well as in *Drosophila* follicles (Overall and Jaffe, 1985) and in ascidian zygotes (Jeffery, 1982). Moreover, in aequorin-loaded medaka eggs, persistent polar zones of high luminescence were visualized more than six years ago. However their meaning has been uncertain because of the possibility that the aequorin rather than free calcium was concentrated at these eggs' poles

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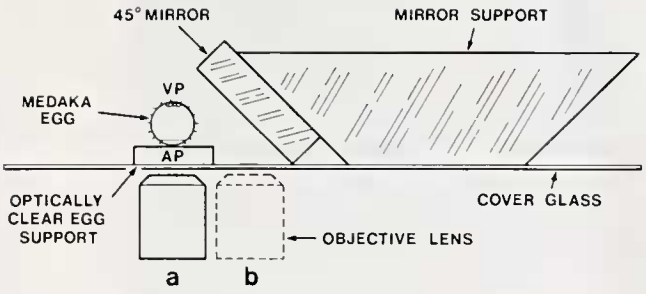


Figure 1. Method of observing an undisturbed medaka egg from either the animal pole (position a) or the side (position b). The stage is moved to attain these two views. VP = vegetal pole; AP = animal pole.

(Jaffe, 1986). In this report we finally show that it is indeed the pattern of free calcium rather than its indicator that creates the observed zones of polar luminescence in such eggs.

Materials and Methods

To obtain gametes, gonads were removed from breeding medaka and placed in a balanced saline solution (BSS: 111 m*M* NaCl; 5.37 m*M* KCl; 1.0 m*M* $CaCl_2$; 0.6 m*M* $MgSO_4$; 5 m*M* HEPES, pH 7.3). To prepare unfertilized eggs for microinjection, we transferred them through five successive washes of Ca^{2+}/Mg^{2+}-free BSS over a period of 1 h. Approximately 1.0 nl of a 0.62% solution of recombinant aequorin (Shimomura *et al.*, 1990) in 100 m*M* KCl, 5 m*M* HEPES, and 0.05 m*M* EDTA was injected equatorially using micropipettes of ≤5 μm tip diameter. To correct for nonuniformities in the distribution of aequorin, we injected other eggs with 1.0 nl of a mixture of 0.45% unmodified recombinant aequorin and 0.15% recombinant that was labeled with one fluorescein per aequorin molecule (Shimomura, 1991). The injection technique employed was Hiramoto's quantitative low-pressure method (Hiramoto, 1962). Details of a procedure used to introduce a micropipette into the thin peripheral cytoplasmic layer of the medaka egg were those described by Gilkey (1983). After microinjection, the eggs were transferred to BSS containing 0.2 m*M* Ca^{2+} for 30 min, transferred to BSS for 30 min, fertilized, transferred to the microscope stage, and observed at 16–17°C.

Two methods were used to observe chorionated eggs. In the first, eggs were placed on a 1-mm thick platform of Dow Corning high vacuum grease and observed in their normal, blastodisc-down orientation with a Zeiss IM-35 inverted microscope, a Nikon Planapo 10/0.45 objective and a 75 mm optical doublet, which together produced a 6× magnified image on the photocathode of an imaging photon detector or IPD made by Imaging Technology Ltd., East Sussex, UK. This device consists of a microchannel plate intensifier with a resistive anode as the positional encoder (Speksnijder *et al.*, 1990b). Eggs were viewed either directly (Fig. 1, lens position "a") or via a mirror (Fig. 1, lens position "b"). The indirect (or side) view enabled us to simultaneously observe both the animal and vegetal poles of the egg. External illumination of the egg for bright field and dark field viewing was achieved with a condenser and a fiber optic cable, respectively. In the second method used to observe the eggs, they were flattened slightly between a coverglass and slide, held with their animal-vegetal axis parallel to the slide, and viewed directly.

To facilitate calcium diffusion and thus dissipate calcium gradients in the ooplasm, we injected eggs with dibromo-BAPTA as follows: a high-pressure system (Medical Systems Corp. PLI-100) was used to microinject 1.5 nl of a solution of dibromo-BAPTA (50 m*M* dibromo-BAPTA; 150 m*M* KCl; 5 m*M* HEPES, pH 7.0) into the equatorial region of an unfertilized egg that had already been injected with aequorin. This egg was then inseminated within 30 s, before the injected buffer had time to block fertilization. [A comparable volume of KCl (150 m*M* KCl; 5 m*M* HEPES, pH 7.0) was injected into control eggs.] If the volume of the ooplasm (except for oil globules) is 46 nl (Fluck, unpub.) and we assume that 60% of the cytosol is water (Bolender, 1978), then we can calculate an accessible ooplasmic volume of 27.6 nl. Thus the dibromo-BAPTA was diluted about 18-fold, to a final concentration in the cytosol of about 2.7 m*M*. At this concentration, this calcium buffer will substantially facilitate the diffusion of Ca^{2+} away from any zones of elevated $[Ca^{2+}]$ which are in the micromolar range (Speksnijder *et al.*, 1989).

The raw data from the IPD system consist of a sequential record of photon positions and times, measured one at a time. This system will record up to around 100,000 photons/s. Because we never encountered more than about 30 photons/s, system saturation was never a problem. Images were generated by accumulating data over any desired interval and representing multiple pho-

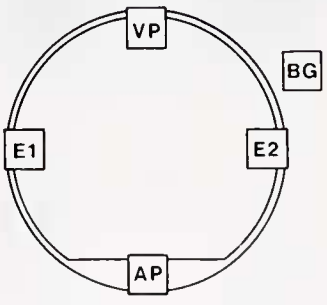
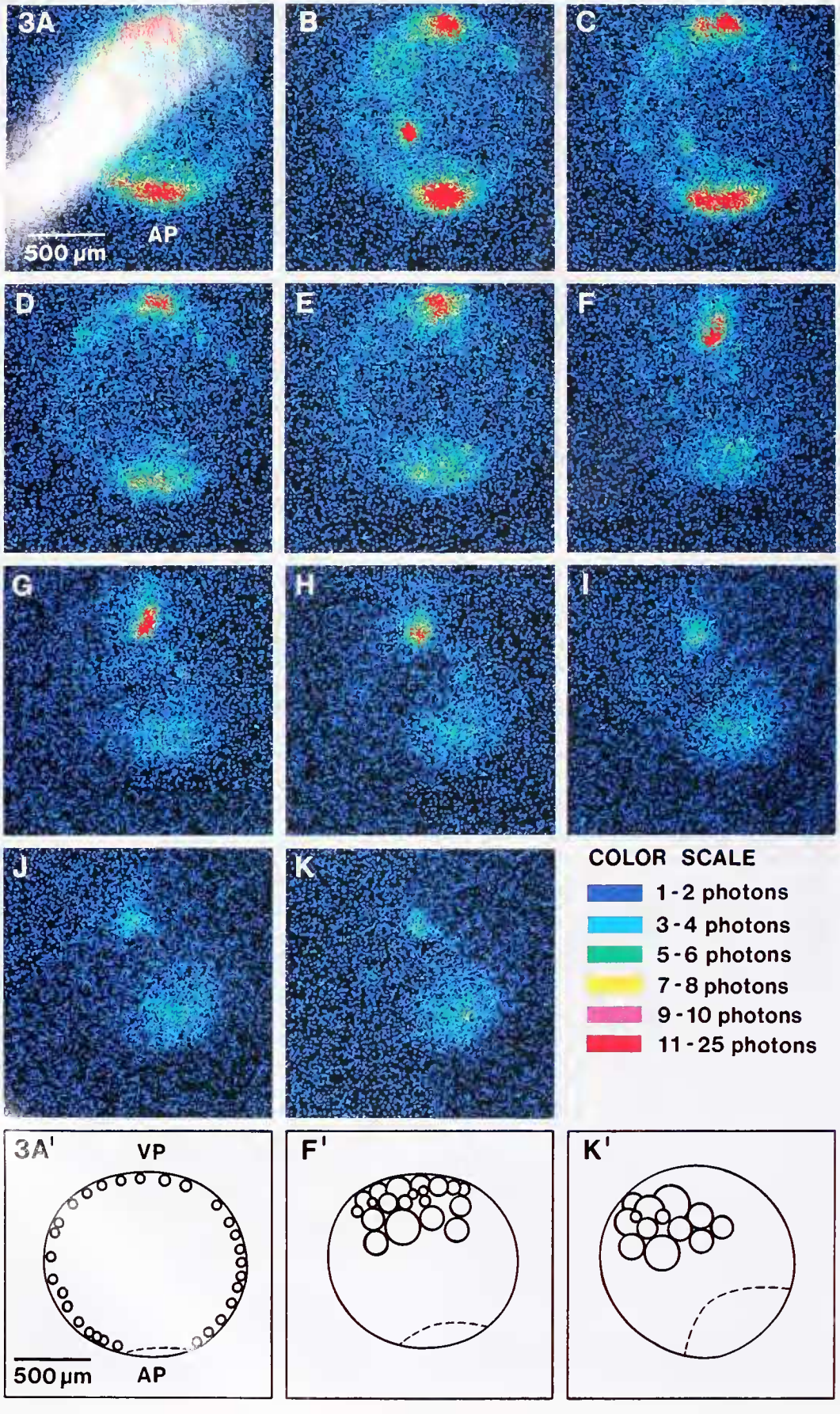


Figure 2. Regions of an aequorin loaded medaka egg's image that were used for a quantitative analysis of its patterns of luminescence and fluorescence. VP = vegetal pole; AP = animal pole; E = equator; BG = background.


3A
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COLOR SCALE
1-2 photons
3-4 photons
5-6 photons
7-8 photons
9-10 photons
11-25 photons
3A'
VP
500 µm
AP
F'
K'

tons per pixel with a color scale. Photon collection was briefly (10 s) interrupted at appropriate intervals to record brightfield images of the eggs. This allowed us to compare luminescent and brightfield images as the relatively slow processes of ooplasmic segregation and cytokinesis proceeded.

We used the IPD system to observe the fluorescence emitted by fluorescein-labeled aequorin in the eggs. Appropriate excitation filters (two 485 DF22 filters in series) and a 530-DF30 barrier filter were obtained from Omega Optical, Inc. (Brattleboro, Vermont). The latter was supplemented with two Schott OG 530 filters.

Our quantitative analysis consisted of measuring luminescence in 150 μm wide square boxes placed at either the vegetal or animal poles and then dividing these values by the mean of those collected from two similar boxes placed at the equator of the egg (Fig. 2). Each resulting luminescence ratio was divided by a corresponding fluorescence ratio (from fluorescein aequorin) to correct for differences in aequorin concentration. All raw light intensity values were corrected for background.

Results and Discussion

Color Figure 3 displays the pattern of luminescence in a representative aequorin injected medaka egg during the first 22 h of development, up to the early to mid gastrula stage. Striking zones of high luminescence persist at both poles throughout this period.

The blastodisc seems to roughly correspond to the animal zone of high luminescence. However, no known structure corresponds to the remarkable, 150 to 300 μm wide vegetal zone of luminescence. Moreover, we do not yet know if the gradual, overall decline in luminescence during the first day of development represents a slow fall in aequorin or in free calcium concentration. However, the observed pattern of luminescence should somehow represent a natural, developmental pattern because medaka eggs, which are loaded with recombinant aequorin, regularly hatch into swimming fish. While roughly consistent with the preliminary results reported earlier (Jaffe, 1986), these new ones should be more reliable because the natural aequorin used in the older studies somehow inhibited development beyond the blastula stage (Ridgway *et al.*, 1977).

Color Figure 4 displays this pattern during the first 2 h, almost to first cleavage; while Figure 7 provides quantitative information as well as temporal detail from fertilization through the first few cleavages. A strong vegetal zone of luminescence appears as soon as the fertilization wave reaches the vegetal pole and remains relatively constant in both intensity and diameter for at least 6 h. However, a distinct animal zone of luminescence does not appear until well after the fertilization wave has subsided. It then slowly oscillates so as to largely disappear and then reappear three times during the first 6 h. These reappearances presumably represent the slow calcium waves that accompany cytokinesis during the first three cleavages (Fluck *et al.*, 1991).

However, the biological role of the first, precleavage zone of animal pole calcium is less clear. The additional examples displayed in Figure 6 indicate that this precleavage zone is a real one that is present between about 20% and 50% of the period between fertilization and first cleavage. The presence of this precleavage zone of animal pole calcium may be somehow connected to the process of ooplasmic segregation because this process is largely completed while this zone is present. Ooplasmic segregation comprises the roughly simultaneous movement of the oil droplets toward the vegetal pole and of the bulk of the cytoplasm to the animal pole. Both oil droplet movement and the thickening of the blastodisc, which indicates cytoplasmic flow, start at about 20% of the precleavage period and are largely over by 70% of it (Fluck *et al.*, unpublished).

Polar luminescence represents foci of calcium not aequorin

Color Figure 5 displays representative patterns of fluorescence coming from eggs injected with fluorescein-aequorin during the precleavage period. No sign of a vegetal focus of aequorin fluorescence can be seen.

Figure 3. Representative patterns of luminescence from an aequorin-loaded medaka egg during the first day of development. Each colored panel shows the luminescence accumulated during two successive hours. Thus panel 3A shows luminescence accumulated for 2 h starting at 5 min after fertilization; 3B shows the luminescence accumulated from 2 to 4 h; 3C, from 4 to 6 h, etc. Panels 3A′, F′, and K′ show sketches of the slowly rotating egg made from brief transmitted light observations carried out just after the corresponding luminescent images were obtained. The persistent antipodal zones of high luminescence remain at the egg's poles.

The luminescence seen in the left hand hemisphere of 3A represents a faint residue of the intense fertilization wave described by Gilkey *et al.* (1978) and by Yoshimoto *et al.* (1986). It is scarcely seen on the right because the aequorin had not yet fully diffused to the right half at this time. The interpolar spot of decreasing luminescence in the left half of panels 3A–C represents calcium leaking in through the slowly healing injection site. It is wound calcium.

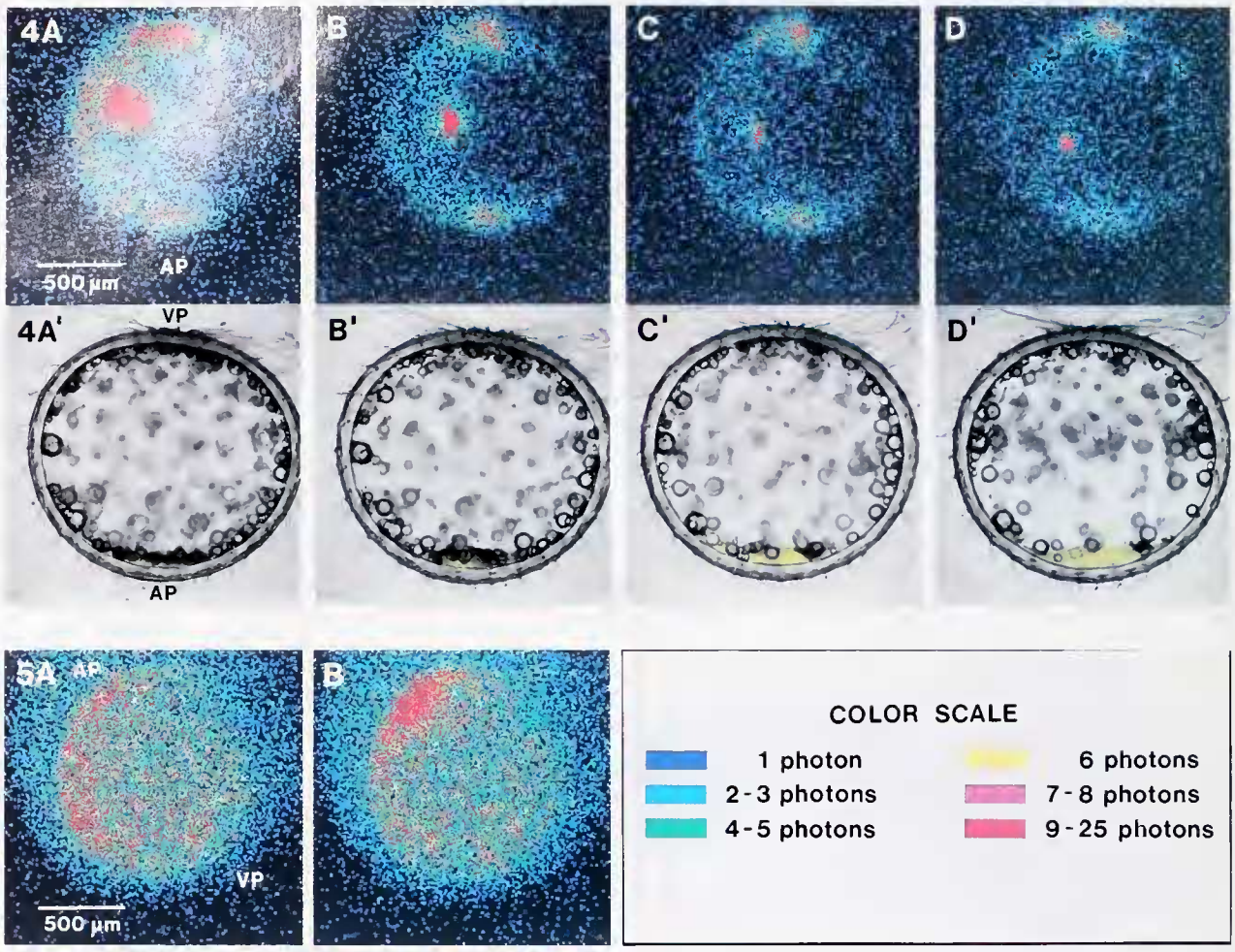

Figure 4. Patterns of luminescence during the first 2 h of development of the same egg shown in Figure 3. Each colored panel represents luminescence accumulated over successive 30-min periods; while each black and white image was obtained via brief transmitted light exposure soon after the corresponding luminescent one. The growing blastodisc's images have been tinted yellow to make them easier to see.

Figure 5. Patterns of fluorescence from a representative medaka egg coinjected with both aequorin and fluorescein-labeled aequorin so as to indicate the distribution of aequorin. A. Observed at 30 min after fertilization, a stage right after accumulation of the image shown in Figure 4A. Note the absence of foci of fluorescence at either pole. The faint, apparent fluorescence seen outside of the egg's boundary is an artifact resulting from incomplete absorption of the exciting light by the barrier filter. (B) Observed 90 min after fertilization, a stage after accumulation of the image shown in Figure 4C. A focus of fluorescence has now appeared at the animal pole because of the accumulation of cytoplasm in the blastodisc during ooplasmic segregation.

In uninjected control eggs, autofluorescence is so low that the egg appears as a black hole against the dim extracellular glow produced by that exciting light that traverses the barrier filter. So a vegetal focus of fluorescence from aequorin is not hidden by autofluorescence. Aequorin is simply not concentrated at the vegetal pole.

Hence the vegetal zone of luminescence must represent a standing zone of high free calcium, not aequorin. The data in Table I confirm this inference quantitatively. Vegetal luminescence remains three times more intense than equatorial or background luminescence when the luminescence ratio is divided by the corresponding ratio of fluorescence coming from coinjected fluorescein-aequorin in the two regions. This inference was further confirmed by the results of experiments in which the eggs were injected with enough of the calcium buffer, dibromo-BAPTA to substantially facilitate the diffusion of Ca^{2+} away from any zones of high $[Ca^{2+}]$, which are in the micromolar range. As Table II shows, such injections halve the relative intensity of the vegetal zone of luminescence.

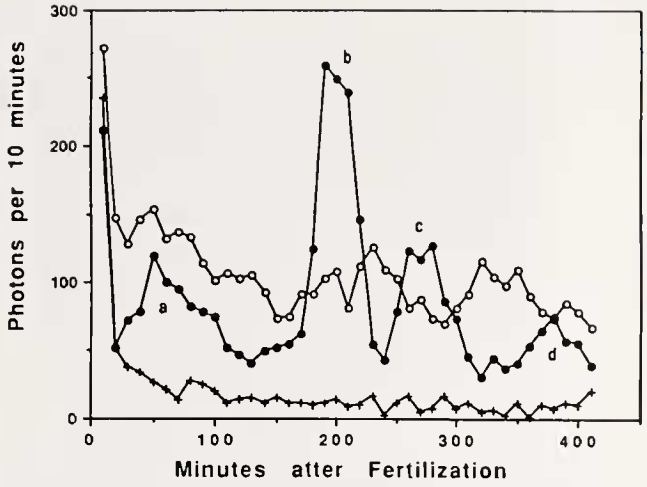

Figure 6. Luminescent intensities observed at the vegetal (○), animal (●), and equatorial (+) regions at various times after fertilization. While the focus of luminescence at the vegetal pole is clearly due to a steady zone of high calcium there, the focus at the animal pole is only clearly above that due to more cytoplasm and hence more aequorin there at the precleavage peak (a) and during each of the first three cleavages (b–d).

Color Figure 5 also displays the pattern of fluorescence coming from fluorescein-aequorin in the animal hemisphere. Here an obvious focus of fluorescence appears at the animal pole together with the precleavage zone of luminescence. So the development of a precleavage zone of high animal calcium is not qualitatively obvious. However, Table I shows that when the ratio of animal pole to equatorial luminescence is corrected for the corresponding fluorescence ratio, it remains about five-fold and thus clearly represents a second zone of high free calcium at the animal pole. Moreover, as Table II and Figure 8 show, the reality of this second, animal zone is also confirmed by the results of coinjecting a calcium buffer. When enough is introduced to substantially facilitate the diffusion of Ca^{2+} away from any zone in the micromolar range, the luminescence at the animal pole falls steadily to a level which is far below the control level.

Significance of the results

This report shows that the zones of high luminescence seen at the poles of aequorin-injected medaka eggs are indeed zones of high free calcium. A preliminary report is available of a similar, if less continuous, high calcium zone at the vegetal pole of *Xenopus* eggs (Miller *et al.*, 1991). Moreover, in ascidian zygotes, striking, periodic calcium waves are regularly seen to start at or near the vegetal pole during the period between fertilization and first polar body formation (Speksnijder *et al.*, 1990b). Because these waves are attenuated en route to the animal pole, they correspond to a high calcium zone at the vegetal pole when averaged over time. This raises the question of how generally high calcium zones appear at the vegetal poles of developing oocytes and eggs.

It also raises a number of other questions. Thus what are the sources (and sinks) which maintain the high calcium zones—particularly the vegetal one—in the medaka egg? To what extent does this calcium come from the medium, from the yolk compartment or from the endoplasmic reticulum? A related question concerns the radial location of these zones. Are they restricted to a very shallow region just under the plasma membrane as they seem to be in fucoid eggs (Jaffe, 1990a) and in medaka cleavage furrows (Fluck *et al.*, 1991)? Above all, what is their role

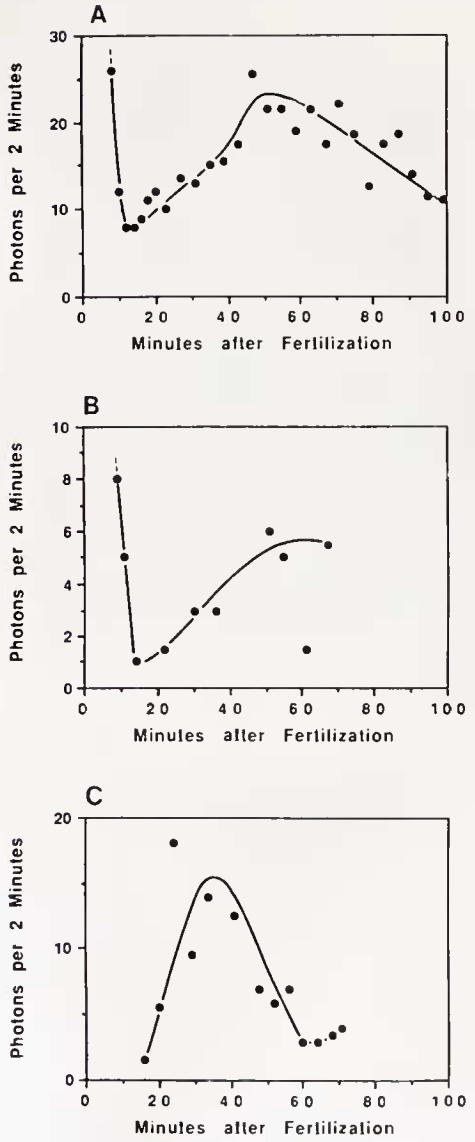

Figure 7. Luminescent intensity observed at the animal pole *versus* time after fertilization in three eggs. (A) The first 100 min of data shown in Figure 7. (B, C) Data from two other eggs.

Relative luminescence [illegible] the equator of aequorin-loaded medaka eggs in the [illegible] period[a]

	N	Luminescence ratio pole/equator ± SEM	
		Vegetal	Animal
Uncorrected:	11	3.0 ± 0.7	8.7 ± 3.4
Corrected for Aequorin Ratio[b]	9	3.4 ± 1.4	4.9 ± 1.6

[a] Luminescence was accumulated between about 15 and 100 min after fertilization at 16°C and thus between 10 and 60% of the period before cleavage.

[b] This was done in a separate group of eggs coinjected with fluorescein-labeled aequorin by dividing the luminescence ratio by the fluorescence ratio.

in development? In particular, what is their role in ooplasmic segregation? Here some evidence is already available. Long ago, Yoshi Sakai reported some striking effects of prick activation on the loci of ooplasmic segregation in medaka eggs (Sakai, 1964). In particular, if vegetal halves are strongly prick-activated, then the cytoplasm accumulates (to form what would normally be the blastodisc) at the point of activation and the oil droplets migrate towards the opposite pole. Such pricking undoubtedly both initiated an activation wave of calcium and established an enduring region of high calcium at the wound (compare Fig. 3 and Fig. 4). It is true that the organization of animal halves proved to be less sensitive to the pricking site. Nevertheless, these old experiments suggest that calcium zones may play at least a reinforcing role in normal ooplasmic segregation. Recently, we have observed that ooplasmic segregation in the medaka egg can be markedly inhibited by injecting enough of the calcium buffer, dibromo-BAPTA, to inhibit the formation of high calcium zones (Miller *et al.*, unpub.).

Table II

Dibromo-BAPTA reduces aequorin luminescence at the animal and vegetal poles of the medaka egg[a]

Injectate(s)	N	Luminescence ratios pole/equator ± SEM	
		Vegetal	Animal
Aequorin	11	3.0 ± 0.7	8.7 ± 3.4
Aequorin + KCl	4	3.0 ± 0.5	7.8 ± 1.9
Aequorin + dbBAPTA	10	1.6 ± 0.3	3.6 ± 0.8

[a] The buffer was injected to a final cytosolic concentration of 2.7 m*M*. Luminescence was accumulated between about 15 and 100 min after fertilization at 16°C and thus between 10 and 60% of the period before cleavage.

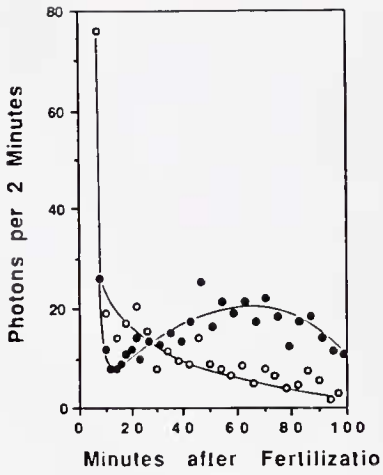

Figure 8. Effect of dibromo-BAPTA injection on the time course of luminescence at the animal pole. Control curve (●) shows data from a single representative egg. Experimental curve (○) shows data pooled from 10 separate eggs.

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